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Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families

Strautnieks, S S ; Byrne, J A ; Pawlikowska, L ; Cebecauerová, D ; Rayner, A ; Dutton, L ; Meier, Y ; Antoniou, A ; Stieger, B ; Arnell, H ; Özçay, F ; Al-Hussaini, H F ; Bassas, A F ; Verkade, H J ; Fischler, B ; Németh, A ; Kotalová, R ; Shneider, B L ; Cielecka-Kuszyk, J ; McClean, P ; Whittington, P F ; Sokal, É ; Jirsa, M ; Wali, S H ; Jankowska, I ; Pawłowska, J ; Mieli-Vergani, G ; Knisely, A S ; Bull, L N ; Thompson, R J

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Severe bile salt export pump deficiency: 82 different *ABCB11* mutations in 109 families

Short title: *ABCB11* mutations in severe BSEP deficiency

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No conflicts of interest exist

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Abbreviations:

ABC, ATP-binding cassette; AFP, α -fetoprotein; BSEP, bile salt export pump; CpG, cytosine-guanine; CC, cholangiocarcinoma; FIC1, familial intrahepatic cholestasis 1; γ -GT, γ -glutamyl transferase; HCC, hepatocellular carcinoma; IC, intracellular loop; MDR1, multidrug resistance protein 1; MDR3, multidrug resistance protein 3; MRP2, multidrug resistance-associated protein 2; NBF, nucleotide-binding fold; OLT, orthotopic liver transplantation; PEBD, partial external biliary diversion; PFIC, progressive familial intrahepatic cholestasis; PCR, polymerase chain reaction; RE, restriction endonuclease; SSCP, single-strand conformation polymorphism; TM, transmembrane domain; UDCA, ursodeoxycholic acid

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Abstract

Background and aims Patients with severe bile salt export pump (BSEP) deficiency present as infants with progressive cholestatic liver disease. We characterised mutations of *ABCB11* (encoding BSEP) in such patients and correlated genotypes with residual protein detection and malignancy risk.

Methods Patients with intrahepatic cholestasis suggestive of BSEP deficiency were investigated by single-strand conformational polymorphism analysis and sequencing of *ABCB11*. Genotypes sorted by likely phenotypic severity were correlated with data on BSEP immunohistochemistry and clinical outcome.

Results Eighty-two different mutations (52 novel) were identified in 109 families (9 nonsense mutations, 10 small insertions and deletions, 15 splice-site changes, 3 whole-gene deletions, 45 missense changes). In 7 families only a single heterozygous mutation was identified despite complete sequence analysis. Thirty-two percent of mutations occurred in >1 family, with E297G and/or D482G present in 58% (52/89) of European families. On immunohistochemical analysis (88 patients), 93% had abnormal or absent BSEP marking. Expression varied most for E297G and D482G, with some BSEP detected in 45% (19/42) of patients with these mutations. Hepatocellular carcinoma or cholangiocarcinoma developed in 15% (19/128) of patients. Two protein-truncating mutations conferred particular risk; 38% (8/21) of such patients developed malignancy versus 10% (11/107) with potentially less severe genotypes (relative risk 3.7 [CL=1.7-8.1, p=0.003]).

Conclusions With this study, >100 *ABCB11* mutations are now identified. Immunohistochemically detectable BSEP is typically absent, or much reduced, in severe disease. BSEP deficiency confers risk of hepatobiliary malignancy. Close surveillance of BSEP-deficient patients retaining their native liver, particularly those carrying 2 null mutations, is essential.

Introduction

Bile salt export pump (BSEP) deficiency is caused by mutations in *ABCB11*^{1, 2}. The severity of BSEP deficiency varies from progressive early-onset¹ to remitting and late-onset phenotypes³⁻⁷. Severe BSEP deficiency falls into the descriptive category of “progressive familial intrahepatic cholestasis” (PFIC)⁸⁻¹², a heterogeneous group of autosomal recessive conditions that disrupt bile formation. BSEP deficiency is among disorders with low serum concentrations of γ -glutamyl transferase (γ -GT) activity despite conjugated hyperbilirubinaemia, as is familial intrahepatic cholestasis 1 (FIC1) deficiency caused by mutations in *ATP8B1*¹³. Both BSEP deficiency and FIC1 deficiency exist worldwide. Their collective estimated Western European incidence is 1/50-70,000 births/year.

BSEP, previously termed “sister of P-glycoprotein”¹⁴, is a member of the ATP-binding cassette (ABC) superfamily and P-glycoprotein / multidrug resistance (MDR/ABCB) subfamily of transporters^{15, 16}. BSEP, expressed at the hepatocyte canalicular membrane, is the major exporter of primary bile acids¹⁷.¹⁹ It actively transports conjugated bile salts into biliary canaliculi against extreme concentration gradients. Liver disease in BSEP deficiency is ascribed to failed secretion and intrahepatocytic accumulation of toxic bile salts. Patients with the progressive form present as infants with high serum bile salts, pruritus, malabsorption, failure to thrive, jaundice and cholestasis. They develop fibrosis and end-stage liver disease before adulthood²⁰⁻²². Partial external biliary diversion (PEBD) and ileal exclusion can relieve pruritus and, in some cases, slow disease progression²³⁻²⁷. However, most patients ultimately need orthotopic liver transplantation (OLT).

We here present the mutations of *ABCB11* in 109 families with severe BSEP deficiency.

Methods

Families

PFIC families were recruited through referral to King's College London or the University of California, San Francisco. All procedures were conducted with informed consent as routine diagnostic investigations or under an institutional-review-board–approved protocol. Referrers supplied clinical data. No patient had elevated serum concentrations of γ -GT activity. Other causes of neonatal hepatitis were excluded, including primary disorders of bile acid synthesis in most cases.

Families were defined as affected by “severe” PFIC and included in the study if at least one family member presented in infancy with a low γ -GT cholestasis that progressed, with clinical and biochemical markers of cholestasis persistently abnormal (absent surgical intervention). They were excluded if cholestasis ever resolved completely.

Selection for likely BSEP deficiency was based on clinical and histological data (specifically: Liver disease without the extrahepatic manifestations (pancreatitis, hearing loss, profound diarrhoea) characteristic of FIC1 deficiency, and exhibiting giant-cell hepatitis rather than bland cholestasis on histological assessment²¹) Where possible, BSEP immunohistochemical analysis (30%)^{2, 28, 29} and/or microsatellite-based haplotype analysis^{13, 30, 31} were used. The resultant subgroup was analysed for *ABCB11* mutations.

One hundred and nine families (data and/or biopsy material available for 132 individuals) met the inclusion criteria of genetically proven severe BSEP deficiency (Supplementary Tables 1A-1E). Eighty-nine families were European (European, Australian, North American), 20 Central Asian/Arab, East Asian (Korean, Japanese, Chinese), South Asian (Indian, Pakistani) or African. BSEP immunohistochemistry was possible for 88 patients; clinical follow-up for malignancy was available in 128. In 7 families, clinical outcome and/or immunohistochemical results from a deceased sibling were included without mutational confirmation. In 5 families only parental DNA was analysed. We have previously reported single

mutations in 8 families^{1, 2, 32}; the second mutant allele is now identified in each. Clinical observations in

21 families have been reported previously^{28, 29, 33}. These families are included to retain our population's mutation profile.

Sequence and genomic structure of ABCB11

The 1321 amino-acid BSEP protein is encoded by *ABCB11* on chromosome 2q24-31. *ABCB11* spans a 108kb genomic region and is composed of 27 coding exons and one 5' untranslated exon (designated 1-28).

ABCB11 cDNA sequence (AF091582; AF136523; NM_003742) and genomic structure¹ are available (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>). Mutation nomenclature³⁴ follows Human Genome Variation Society recommendations (<http://www.hgvs.org/mutnomen/>). Previously published mutations have been revised accordingly.

Mutation detection strategy

Patients were initially screened by restriction-endonuclease (RE) digestion for recurrent changes at mutation hotspots and, depending on ethnicity, for population-associated changes. *ABCB11* exons subsequently underwent single-strand conformation polymorphism (SSCP) analysis in 44 patients followed by sequence analysis of exons with identified mobility changes. Latterly this was replaced by primary sequencing. All exons were sequenced until clearly damaging, or previously known, mutations were identified on both alleles. Samples with novel missense changes were sequenced throughout. Mutations were confirmed using freshly extracted DNA from affected individuals and parental DNA (as available). Missense changes were distinguished from polymorphisms by several criteria. First was their absence from ethnically matched control panels of at least 300 alleles (published³⁵⁻³⁸, in public databases [<http://pharmacogenetics.ucsf.edu/>]³⁹, or within this study). Also considered was conservation across BSEP orthologues and MDR/ABCB homologues. Finally, predicted functional effects and differences in physical and chemical properties were assessed.

Statistics

The Statistics Calculator was used (<http://www.cebim.utoronto.ca/practise/ca/statscal/>). Chi-squared testing assessed differences between groups. Using the same data and calculator, relative risk and 95% confidence limits were similarly calculated.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded liver, when available, were routinely stained and immunostained for BSEP using a polyclonal antibody raised in rabbit to the carboxy-terminal 21 amino acids of BSEP as previously described¹⁹. As a control for antigen preservation or protein-expression deficiencies not specific to BSEP, parallel sections were immunostained for a structurally similar canalicular ABC transporter, multidrug resistance-associated protein 2, using a monoclonal antibody raised in mouse (Signet/Bioquote, York, UK). Findings were evaluated by light microscopy as described^{28, 29}. For 6 families immunohistochemical protocols used were as described². BSEP marking was classified as normal, not detected or abnormal, where abnormal refers to either reduced intensity or focal absence. Immunohistochemical analysis preceded genetic analysis in 30% of cases and followed it in the remainder. Abnormality was judged by two or more investigators, all blinded to genetic status.

DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted from blood and tissue samples using standard protocols. PCR amplification was conducted using *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) and Roche Fast Start PCR systems (Roche Diagnostics, Basel, Switzerland). Primer details provided in Supplementary Table 2.

Microsatellite-marker typing Microsatellite-marker haplotype analysis was conducted across the *ABCB11* (2q24) and *ATP8B1* (18q21) chromosomal regions in consanguineous families, or those with ≥ 2 affected children, to determine which gene was likely mutated. Marker loci were selected from genetic maps⁴⁰ and developed from polymorphic repeats (Human Genome Mapping Project/Celera reference sequences; <http://www.ncbi.nlm.nih.gov/>). Primers were designed to allow multiplex analysis based on product size

and fluorescent label (Supplementary Tables 3A/3B). Amplification products were separated on a 3100

Avant Genetic Analyser, data were analysed using GeneMapper software (all Applied Biosystems, Foster City, CA) and haplotypes were constructed. Families with non-Mendelian segregation of mutations were investigated for deletions of the *ABCB11* chromosomal region using microsatellite markers spanning 16.2Mb of chromosome 2.

Restriction-endonuclease digestion

RE digestion was used to identify common or recurring changes rapidly and to screen ethnically matched control panels for novel changes. Enzymes were selected using NEBcutter V2.0 (<http://tools.neb.com/NEBcutter2/index.php>; New England Biolabs or Roche Diagnostics). The common mutations E297G, D482G, R575X, R1153C and R1153H abolish *HphI*, *FokI*, *FokI*, *BsrBI* and *BsrBI* sites respectively, whilst G982R creates an *AlwNI* site. PCR-amplified exon-digestion products underwent 35% agarose gel electrophoresis (Supplementary Table 4).

Single-strand conformation polymorphism analysis

SSCP analysis was conducted using 12.5% acrylamide GeneGel Excel nondenaturing gels on a GenePhor Electrophoresis system (all Amersham Biosciences, Little Chalfont, UK), initially at 5°C and, if necessary for enhanced resolution, at 15°C. SSCP patterns were visualised by DNA silver staining (Amersham Biosciences). Products larger than 150bp were digested before analysis (as above).

Sequencing

PCR products were purified using the High Pure PCR purification system (Roche Diagnostics) before direct sequence analysis using the version 3.1 Dye Terminator cycle sequencing kit (Applied Biosystems) and electrophoresis on a 3100 Avant Genetic Analyzer. Data were analysed using Sequencher (Gene Codes, Ann Arbor, MI) or SeqScape (Applied Biosystems) software.

Results

Eighty-two different *ABCB11* mutations were identified on 208 alleles in 109 families with severe BSEP deficiency (Tables 1-3; Figure 1; Supplementary Tables 1A-1E). Homozygosity, or compound heterozygosity, for *ABCB11* mutations was completely concordant with disease expression in all families genotyped. Fifty-two mutations were novel. Eighteen previously reported severe mutations were not detected^{1, 2, 5, 44-47}.

The 82 mutations identified (Tables 1-3, Figure 1) included 9 (11%; 4 novel) nonsense mutations, 10 (12%; 8 novel) small insertions and deletions, and 15 mutations (18%; 6 novel) predicted to disrupt premRNA splicing. Sixteen (15%), 15 (14%) and 27 (25%) of the 109 families respectively, carried at least one such change.

Whole-gene deletions occurred on a single allele in 3 families. The affected individuals in families 12 and 51 appeared homozygous for a paternal mutation, the child in family 11 for a maternal mutation. Deletions confirmed and sized by haplotype analysis across the *ABCB11* chromosomal region accounted for apparent homozygosity. In family 12 the flanking markers were *D2S156* and *D2S326*; the deletion included up to 12.5Mb of sequence²⁹. In family 51 the deletion occurred between markers *D2S399* and *LRP2* (encoding low density lipoprotein-related protein 2) and included up to 2.24Mb of sequence. Exact breakpoints were not determined but both lay outside the coding and promoter regions of *ABCB11*. Extended haplotype analysis was not possible in family 11.

The remaining 55% of mutations were missense substitutions (Table 3, Figure 1), which were present on at least one allele in 86 (79%) families. Forty-five different mutations (32 novel) were identified.

Thirty-two percent (26) of the 82 mutations occurred, or had been reported to occur, in ≥ 2 families, with 16% (13) in ≥ 3 families. Most frequent were E297G and D482G, one or both of which were present in 58% (52/89) of European families, and 15% (3/20) non-European families. E297G was detected in 34

European families (41 alleles) and on one allele in both an African-American and a South Asian family.

D482G occurred in 20 European families (25 alleles) and on one allele in a Central Asian/Arab family.

In all but 10 families mutations were identified on both alleles. Of the remainder, in 2 families (107, 109), only maternal DNA was available; in one (108) available material was insufficient for complete sequence analysis; and in 7 (100-106) a second mutation was not detected despite complete sequence analysis.

Of 99 families in which complete sequence analysis was possible and mutations were identified on both alleles patients were homozygous for a single mutation in 36% (36/99) of cases and compound heterozygotes for 2 different mutations in the remaining 64% (63/99). In 23 families homozygosity was associated with known consanguinity, whilst in 9 families, 2 copies of either E297G or D482G were found.

To assess effects of specific *ABCB11* genotypes on expression of immunohistochemically detectable BSEP protein, families were grouped according to whether they carried: 2 likely protein-truncating mutations; at least one missense mutation (E297G or D482G excluded); at least one copy of E297G; at least one copy of D482G; or only one identified mutation (Supplementary Tables 1A-1E).

Immunohistochemical analysis was possible in 88 patients. All evaluated patients with 2 predicted protein-truncating mutations lacked demonstrable BSEP (Supplementary Table 1A). Variability in BSEP expression was greatest when either of the 2 common European mutations, E297G or D482G, was present on one or both alleles (Supplementary Tables 1C-1E).

Twenty-nine patients with at least one copy of E297G were immunostained: BSEP was not detected in 16 (55%), was deficient in 12 (41%), and normal in one. For 14 patients with at least one copy of D482G, BSEP was not detected in 8 (57%), was abnormal in 3 (21%) and normal in 3 (21%). In total 45% (19) of 42 immunostained patients with either of these mutations exhibited some BSEP expression. When all genotypes were considered, BSEP marking was absent in 72% (63/88), abnormal in 22%

(19/88) and normal in 7% (6/88) of patients. Thus in total 93% (82/88) of all immunostained patients had abnormal or absent BSEP marking. Representative patterns are illustrated in Figures 2A-H.

Outcome data (Supplementary Tables 1A-1E) confirmed that patients with BSEP deficiency are at considerable risk of hepatobiliary malignancy. Fifteen percent (19/128) of evaluable patients developed hepatocellular carcinoma or cholangiocarcinoma. Correlation with genotype identified those with 2 protein-truncating mutations as being at particular risk. Thirty-eight percent (8/21) of patients with 2 predicted protein-truncating mutations developed malignancy versus 10% (11/107) of patients with potentially less severe defects, giving a relative risk of 3.7 (CL=1.7-8.1 p=0.003)

Discussion

In keeping with the severe phenotype for which study subjects were selected, at least 45% (37) of the 82 different mutations identified were predicted to result in premature protein truncation or failure of protein production (Tables 1, 2). Of the 10 deletions and insertions identified, 8 resulted in a frameshift and the introduction of a premature termination codon, whilst the other 2, in-frame deletions of 4 and 7 amino acid residues, were likely to lead to protein misfolding and degradation. Deletions were more frequent than insertions and typically involved the loss of one or 2 nucleotides. They were uniformly distributed throughout the gene (Figure 1). Most occurred within repeats or strings of nucleotides, suggesting origin by slippage or misalignment during DNA replication. The exception was exon 11, with 4 different deletions, 3 identified in the current study and one previously⁴⁴. Of these, 3 arose between nucleotides 1100-1146, suggesting a deletion hotspot.

Of the 15 splice-site changes identified, all but 2 involved the invariant GT or AG dinucleotides respectively at positions +1 / +2 of the 5' donor and -2 / -1 of the 3' acceptor splice sites. The change c.1435-13_1435-8del is also predicted to be damaging. The remaining change, c.2012-8T>G, disrupted the pyrimidine tract of the already poorly conserved 3' splice site of intron 16. Analysis of liver cDNA from a patient with this mutation (64) demonstrated skipping of exon 17 (unpublished data).

The most common defects were missense mutations. Forty-five different substitutions were identified, with a missense mutation present on at least one allele in 79% (86/109) of families. However, whilst insertions, deletions, nonsense and splicing mutations are readily envisaged as damaging, the effect of a missense substitution is more difficult to predict. Changes in amino acid size, charge, polarity and hydrophobicity can all disrupt functional domains, protein structure, or affect protein processing and trafficking. At the nucleotide level, changes within the coding region may disrupt sequences that enhance or repress pre-mRNA splicing⁵⁰.

Substitutions were considered detrimental based on usual criteria (*cf.* Methods, mutation-detection strategy). In all but one case substitutions were not present in control panels. The exception was E297G, present on a single allele in 1/200 European Caucasian control chromosomes (<http://pharmacogenetics.ucsf.edu>)³⁹, in keeping with the high frequency of this allele among European BSEP-deficiency patients.

Degrees of conservation across orthologous and homologous proteins indicate the likely importance of a given amino acid. All altered residues were conserved or, in 2 cases, replaced only by conservative substitutions, in BSEP orthologues in the mouse, rat and skate (Table 3). Eighty-seven percent of missense residues were conserved or substituted only conservatively in homologous MDR/ABCB family members (multidrug resistance 1 [MDR1] and multidrug resistance 3 [MDR3] proteins).

At the biophysical level, 89% of missense mutations were predicted to change at least one aspect among charge, secondary- or tertiary-structure interactions, and hydrophobicity/polarity of BSEP (Table 3)⁵¹. Sixty percent of mutations significantly changed amino-acid residue charge, 51% size, and 69% hydrophobicity/polarity. Forty-three percent introduced or removed residues whose interactions typically determine secondary and tertiary protein structure. Of these, 22% introduced disulfide-bridge - forming cysteines or alpha-helix - breaking prolines; the remainder introduced or removed the hydroxylated residues serine or threonine.

Whilst protein-truncating mutations were distributed uniformly throughout the protein (Figure 1), 60% of missense changes clustered in the 2 highly conserved NBF domains (residues 414-610, 1072-1321⁵²; 38% in NBF1 and 22% in NBF2). Fourteen changes (31%) occurred within, or immediately adjacent to, the Walker motifs. Of the remainder, 7 (16%) occurred in the transmembrane (TM) domains; 5 of these introduced a charged residue into a hydrophobic domain and, in 3 cases, simultaneously removed glycines, which stabilise alpha-helical TM spans. The intracellular loops contained 6 changes (13%), of

which 3 including E297G, occurred in intracellular loop 2. No changes were identified in the extracellular loops although 2 have been reported^{2, 44}.

In the 93% (99/106) of families in which complete sequence analysis was possible, 2 mutant *ABCB11* alleles were identified. In the remaining 7 families only a single mutation was identified despite extensive analysis. In 5 cases the single mutations were splice site changes or E297G/D482G. In only 2 families were novel missense mutations identified (Q466K, N490D). Consistent with pathogenicity, both were at conserved sites in homologues and were absent from control populations. Q466K was associated with abnormal BSEP marking (detrimental). N490D was associated with normal marking; however, this mutation lies within NBF1, a region in which disease-associated mutations co-existed with retained BSEP marking (see below). Whilst we cannot exclude mutations in genes other than *ABCB11* in this patient, analysis of *ATP8B1* has identified no defects (unpublished data).

Most cystic fibrosis patients in whom sequencing detects only one mutation harbour microdeletions of one or more exons⁵³. This is likely also true for *ABCB11*. Such deletions, unless homozygous, will not be detected by genomic sequencing; exon-dosage analysis such as multiplex ligation-dependent probe amplification must be employed.

Among the 82 different mutations identified in this study, 32% (26) occurred, or had been reported, in multiple families, with 16% (13) present in ≥ 3 families. These likely represent both recurrent and founder mutations. The most common natural mutation hotspots are cytosine-guanine (CpG) dinucleotides⁵⁴.

Thirty-three percent (18) of 54 missense and nonsense mutations occurred at these sites (Tables 1 and 3).

Ten mutations occurred in multiple families: R470Q, R832C³³, R948C, A1110E and R1231Q⁴⁸ have now been reported in 2 families; R1090X² in 3 families; G982R^{1, 2}, R1153C^{1, 47} and R1153H in 4 families; and R575X in 6 families^{1, 2, 32, 45}.

Six common missense and nonsense changes occurred at non-CpG sites: R520X and A588V³³ in 2 European families and E1302X and I541L^{33, 49} in 3 European families each. By far most common, however, were E297G and D482G, at least one of which was present in 58% (52/89) of European and in 15% (3/20) of non-European families. The population distribution of E297G, most frequent along the North European seaboard, suggests origin in Northern Europe and spread southwards through Central and Eastern Europe. The mutation was also found in one South Asian and one African-American family. In contrast, D482G likely originated in Central or Eastern Europe, with cases identified in Polish, Austrian, Slovak, Czech, Hungarian and Russian families. This mutation was also present in a Greek and an Iranian family.

Five common insertions and deletions were identified, with c.379delA in 3 apparently unrelated Arab families and c.1145-1165del, c.1583_1584delTA, c.1941delA²⁸ and c.2787_2788insGAGAT⁵ in 2 European families each. Five common splice-site mutations occurred, with c.611+1G>A²⁸; c.20128T>G²⁸; c.2178+1G>A^{4, 28}; c.2343+1G>T²⁹ and c.3213+1delG² in respectively 2, 7, 4, 3 and 2 families. Geographical distributions suggest that most are likely founder mutations.

Mutation clusters were also observed. For NBF domains this likely reflects functional importance. At other sites both mutations and polymorphisms clustered within the same or adjacent codons, suggesting sequence instability or mutagen interaction. Mutation clusters include Y472C, Y472X²⁸ and I541L^{33, 49} / I541T. Four different changes occurred at, or adjacent to, the 5' splice site of intron 9: R303K, c.908+1delG^{1, 33}, c.908+1G>T²⁸ and c.908+1G>A. Three occurred at the 5' splice site of intron 18: c.2178+1G>A^{4, 28}, c.2178+1G>T and c.2178+1G>C.

BSEP deficiency represents a phenotypic continuum between progressive early-onset¹ disease and remitting, and late-onset, phenotypes³⁻⁷. Eleven different mutations have been reported in BSEP deficiency disease clinically assessed as intermediate⁶ or mild in severity^{4,5,7}. Three of these, E297G, A570T, and c.2178+1G>A, have also been found in PFIC. In milder disease, missense mutations

predominate over those that result in protein truncation or a failure of protein production; more mutations occur in less conserved regions, e.g., intracellular loops, than in the NBFs; and mutated residues are less likely to be conserved. Compound heterozygosity for both a severe and a mild mutation may result in mild disease. That homozygosity for E297G is seen in discrepant phenotypes strongly indicates that environment and genetic background also play roles.

Immunohistochemistry appears valuable in identifying likely BSEP deficiency, as many patients studied exhibited little or no detectable BSEP at the hepatocyte canaliculus^{2, 28, 29}. An anti-C-terminal antibody can be expected not to mark when protein is absent or when a misfolded or truncated “partial protein” is expressed. Immunoreactivity may depend on tissue processing, disease stage, therapeutic intervention and disease mechanism. These factors are to be considered in interpreting immunohistochemical results.

Immunohistochemically detectable BSEP expression does not exclude functional BSEP deficiency. Twenty-eight percent (25/88) of patients analysed exhibited some degree of BSEP marking; in 6 expression was considered normal. Residual marking was most striking in patients with E297G or D482G, with some seen in 45% (19/42) of patients carrying at least one of these alleles. Ten additional missense mutations were associated with detectable BSEP marking (Supplementary Tables 1B-1E, Figures 2A-2H). Abnormal marking was seen with L50S, Q466K, N515T, R517H, I541L, and F548Y, and normal with N490D, G562D, R832C, and A1110E. As most of these were found in combination with E297G or D482G their individual effects could not be assessed. Nine of the 12 mutations occurred within the highly conserved NBFs (8 within NBF1, 4 within or adjacent to Walker motifs) suggesting an effect on protein function. Abnormal (typically reduced) marking may also result from decreased protein production or defective sorting or instability of an otherwise functional protein. Such patients in particular may be amenable to therapeutic interventions such as PEBD or the use of pharmacological agents which enhance BSEP cell-surface expression, e.g., 4-phenylbutyrate⁵⁵.

The above notwithstanding not to detect BSEP immunohistochemically, or to demonstrate BSEP deficiency, is highly useful. Deficiency or absence of BSEP expression could be demonstrated in 93% (82) of the 88 patients in whom immunohistochemical study was possible. As some patients were selected for mutational analysis in part because they lacked immunohistochemically detectable BSEP, the exact sensitivity of this technique could not, however, be assessed.

We have previously shown that BSEP deficiency is associated with increased susceptibility to hepatobiliary malignancy^{28, 29}. In this study 38% (8/21) of patients with 2 predicted protein-truncating mutations (Supplementary Table 1A) developed either hepatocellular carcinoma or cholangiocarcinoma, versus 10% (11/107) of other patients (Supplementary Tables 1B-1E), giving a relative risk of 3.7 (1.78.1, p=0.003). Of the other 13 patients with 2 protein-truncating mutations, all but 3 have undergone OLT or died of non-malignant disease. The 3 who retain their native livers (ages 2.4yrs, 7yrs and 16yrs) are under close observation. Without OLT the incidence of malignancy in patients with 2 protein-truncating *ABCB11* mutations is expected to exceed that observed in this study. BSEP-deficient patients, in particular those carrying 2 predicted null mutations, who retain their native liver require close surveillance for hepatobiliary malignancy.

Legends

Table 1: Nonsense mutations, deletions and insertions

Abbreviations: EU, European Caucasian; CA/AR, Central Asian/Arab; EA, East Asian; AF, African.

Table 2: Splice site mutations

Abbreviations: EU, European Caucasian; CA/AR, Central Asian/Arab. *Denotes studies in which mutations are associated with mild disease.

Table 3: Missense mutations

Abbreviations: EU, European Caucasian; CA/AR, Central Asian/Arab; EA, East Asian; SA, South Asian; AF, African; m/r/s bsep, mouse/rat/skate bsep; IC, intracellular loop; Hyd/Pol, hydrophobicity or polarity; NBF, nucleotide-binding fold; NBF/TM, location between NBF domain and TM region; NH2 Term, amino terminal; MDR1/3, multidrug resistance proteins 1/3; TM, transmembrane domain. Unless specified all proteins are human. *Denotes studies in which mutations are associated with mild disease.

Figure 1: Schematic representation of BSEP with location of mutations identified in this study. BSEP is a full ABC transporter⁴¹ composed of 2 homologous halves, arranged in tandem and joined by a linker region. Each half comprises a hydrophobic membrane domain, containing 6 hydrophobic transmembrane (TM) spans, and a cytoplasmic nucleotide binding fold (NBF). The NBFs contain the Walker A/B motifs, characteristic of all nucleotide-binding proteins, and ABC signature, stacking aromatic, D, H and Q, loops which define ABC proteins⁴². The NBFs bind and hydrolyse ATP to generate transport-driving energy, whilst the TM domains form the pore and define substrate specificity. Schematic generated using TOPO2 software (<http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl>). Protein topology predicted by comparison with MDR1⁴³. TM spans predicted to fit the accepted topology using TopPred II (<http://www.expasy.ch/tools/>). Singly mutated residues represented as coloured residues; founder/recurrent mutations indicated by arrows. For insertions/deletions the affected residue

is indicated, for splice sites the adjacent amino acid. Key: Blue-missense; Red-stop; Green

deletion/insertion; Orange-splice-site; Black-several different mutations at this site; Purple-Walker A, B and ABC motifs.

Figure 2: Expression patterns on immunostaining for canalicular transporters bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2). Avidin/biotin technique; all sections counterstained with haematoxylin.

A. Normal BSEP marking of control liver without cholestasis. Anti-BSEP antibody marks an orderly canalicular network. Original magnification 200x.

B. Normal MRP2 marking of control liver without cholestasis. Anti-MRP2 antibody marks an orderly canalicular network. Original magnification 200x.

C. Absent BSEP marking in patient 4, homozygote for *ABCB11* mutation yielding Y472X. Hepatectomy for cirrhosis with hepatocellular carcinoma, age 1 year. No marking for BSEP is seen. Original magnification 200x.

D. MRP2 marking in patient 4 (see C, above). Anti-MRP2 antibody highlights canalicular network. Original magnification 200x.

E. BSEP marking in patient 29, homozygote for *ABCB11* mutation yielding R832C. Hepatectomy for cirrhosis, age 3 years. The canalicular network is delineated well. Original magnification 200x.

F. MRP2 marking in patient 29 (see E, above). Anti-MRP2 antibody highlights canalicular network. Original magnification 200x.

G. BSEP marking in patient 47b, compound heterozygote for *ABCB11* mutations yielding L50S and A1110E. Hepatectomy for cirrhosis with hepatocellular carcinoma, age 6 years. Canalicular marking for BSEP is abnormal; it is present only focally (arrows) and is assessed as both faint and patchy. Original magnification 200x.

H. MRP2 marking in patient 47b (see G, above). Reaction product (anti-MRP2 antibody) highlights centres of hepatocellular rosettes as well as canalicular network. Hepatectomy. Original magnification 200x.

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Nucleotide change	Exon	Predicted effect	Mutation type	CpG Site	Total number of families in which identified	Families in current study	E o
c.74C>A	2	p.Ser25X	Nonsense	No	1	18	E
c.379delA	5	p.Thr127HisfsX6	Deletion		3	1-3	C
c.1101_1102delAG	11	p.Val368ArgfsX27	Deletion		1	52	E
c.1139delT	11	p.Leu380TrpfsX18	Deletion		1	13	E
c.1145_1165del	11	p.Ala382_Ala388del	Deletion		2	14, 15	E
c.1416T>A	13	p.Tyr472X	Nonsense	No	1	4	C
c.1558A>T	14	p.Arg520X	Nonsense	No	2	59, 81	E
c.1583_1584delTA	14	p.Ile528SerfsX21	Deletion		2	53, 54	E
c.1723C>T	15	p.Arg575X	Nonsense	Yes	6	12, 13, 17, 19, 37	E
c.1941delA	16	p.Gly648ValfsX6	Deletion		2	16, 55	E
c.2316T>G	19	p.Tyr772X	Nonsense	No	1	5	E
c.2787_2788insGAGAT	22	p.Lys930GlufsX79	Insertion		2	36	E

c.2906_2917del	23	p.Lys969_Lys972del	Deletion		1	56	E
c.3268C>T	25	p.Arg1090X	Nonsense	Yes	3	17, 60	E
c.3438delA	26	p.Val1147X	Deletion		1	57	E
c.3491delT	26	p.Val1164GlyfsX7	Deletion		1	58	E
c.3643C>T	27	p.Gln1215X	Nonsense	No	1	38	E
c.3703C>T	27	p.Arg1235X	Nonsense	Yes	1	6	E
c.3904G>T	28	p.Glu1302X	Nonsense	No	3	20, 21, 61	E

Table 1. Nonsense mutations, deletions and insertions in *ABCD1*.

Splice site	Nucleotide change	Exon skippable in frame	Total number of families in which identified	Families in current study	Ethnic origin	Previous reports
3' Intron 5	c.390-1G>A	No	1	8	CA / AR	
5' Intron 7	c.611+1G>A	No	2	21, 62	EU	28
5' Intron 9	c.908+1delG	No	1	39	EU	1, 33
5' Intron 9	c.908+1G>T	No	1	107	EU	28
5' Intron 9	c.908+1G>A	No	1	82	CA / AR	
3' Intron 13	c.1435-13_1435-8del	Yes	1	63	EU	28
3' Intron 16	c.2012-8T>G	No	7	14-16, 40, 64, 65, 100,	EU	28
5' Intron 18	c.2178+1G>T	No	1	19	EU	
5' Intron 18	c.2178+1G>A	No	4	18, 20, 41, 42	EU	4*, 28
5' Intron 18	c.2178+1G>C	No	1	43	EU	
3' Intron 18	c.2179-2A>G	Yes	1	11	EU	
5' Intron 19	c.2343+1G>T	Yes	3	44, 66, 67	EU	29
5' Intron 19	c.2343+2T>C	Yes	1	9	EU	28
3' Intron 21	c.2611-2A>T	Yes	1	10	CA / AR	
5' Intron 24	c.3213+1delG	No	2	7	EU, CA / AR	2

Table 2: Splice site mutations in *ABCB1*

Nucleotide change	Predicted effect	Exon	CpG site	Location	Change in: Size Charge Hyd/Pol Shape				Conservation BSEP mbs epr bseps bsep	M M D D R R 1 3	Total number of families in which identified	Fam curre
c.149T>C	p.Leu50Ser	4	No	NH2 Term	Y		Y	Y	L L L L	M L	1	47
c.470A>G	p.Tyr157Cys	6	No	TM2	Y		Y	Y	Y Y Y Y	Y Y	1	49
c.725C>T	p.Thr242Ile	8	No	TM4	Y		Y		T T T T	T T	1	41
c.890A>G	p.Glu297Gly	9	No	IC2	Y	Y	Y		E E E E	Q Q	67	51-80, 102-10 109
c.908G>A	p.Arg303Lys	9	No	IC2					R R R R	R N	1	22
c.937C>A	p.Arg313Ser	10	Yes	IC2	Y	Y	Y	Y	R R R R	R E	1	83
c.980G>A	p.Gly327Glu	10	No	TM5	Y	Y	Y		G G G G	G G	1	50
c.1168G>C	p.Ala390Pro	11	No	TM / NBF				Y	A A A A	A A	1	68
c.1229G>A	p.Gly410Asp	12	No	TM / NBF		Y	Y		G G G G	G G	1	84
c.1238T>G	p.Leu413Trp	12	No	TM / NBF					L L L L	P P	1	45
c.1388C>T	p.Thr463Ile	13	No	Adj Walker A	Y		Y	Y	T T T T	T T	1	38

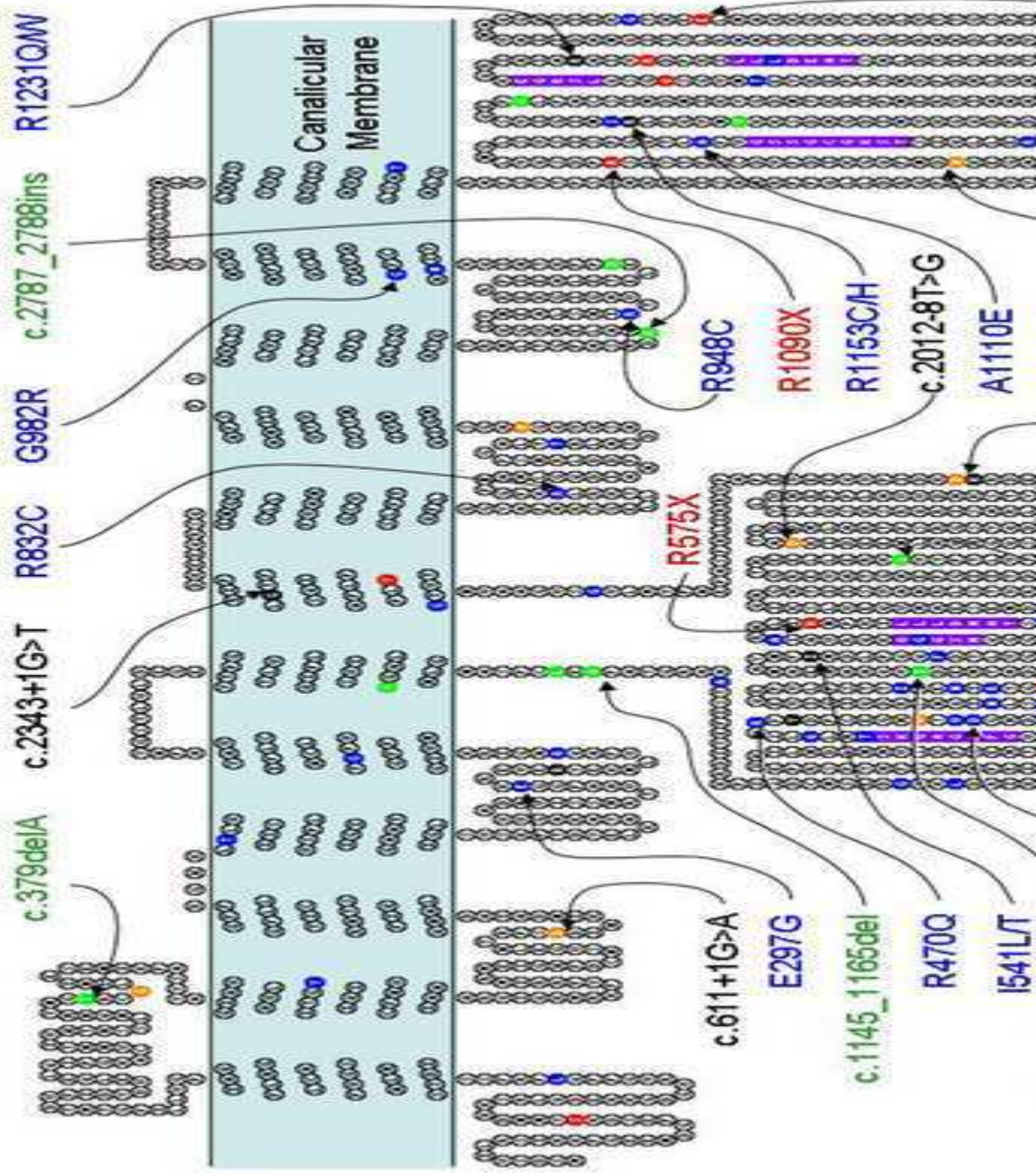
c.1396C>A	p.Gln466Lys	13	No	Adj Walker A		Y			Q Q Q E Q Q	1	101
c.1409G>A	p.Arg470Gln	13	Yes	Adj Walker A		Y			R R R R R R	2	23, 85
c.1415A>G	p.Tyr472Cys	13	No	Adj Walker A	Y		Y	Y	Y Y Y Y Y Y	1	46
c.1442T>A	p.Val481Glu	14	No	NBF1	Y	Y	Y		V L L L V I	1	36
c.1445A>G	p.Asp482Gly	14	No	NBF1		Y	Y		D D D D D D	22	81– 99
c.1460G>C	p.Arg487Pro	14	Yes	NBF1	Y	Y	Y	Y	R R R R R R	1	24
c.1468A>G	p.Asn490Asp	14	No	NBF1		Y			N N N N N N	1	106
c.1535T>C	p.Ile512Thr	14	No	NBF1	Y		Y	Y	I I I I I I	1	46
c.1544A>C	p.Asn515Thr	14	No	NBF1			Y	Y	N N N N N N	1	86
c.1550G>A	p.Arg517His	14	Yes	NBF1		Y	Y		R R R R R R	1	87
c.1621A>C	p.Ile541Leu	14	No	NBF1					I I I I I I	3	25, 39
c.1622T>C	p.Ile541Thr	14	No	NBF1	Y		Y	Y	I I I I I I	1	44
c.1643T>A	p.Phe548Tyr	15	No	Adj ABC					F F F F F F	1	69
c.1685G>A	p.Gly562Asp	15	No	ABC		Y	Y		G G G G G G	1	88
c.1708G>A	p.Ala570Thr	15	Yes	ABC / Walker B				Y	A A A A A A	1	26

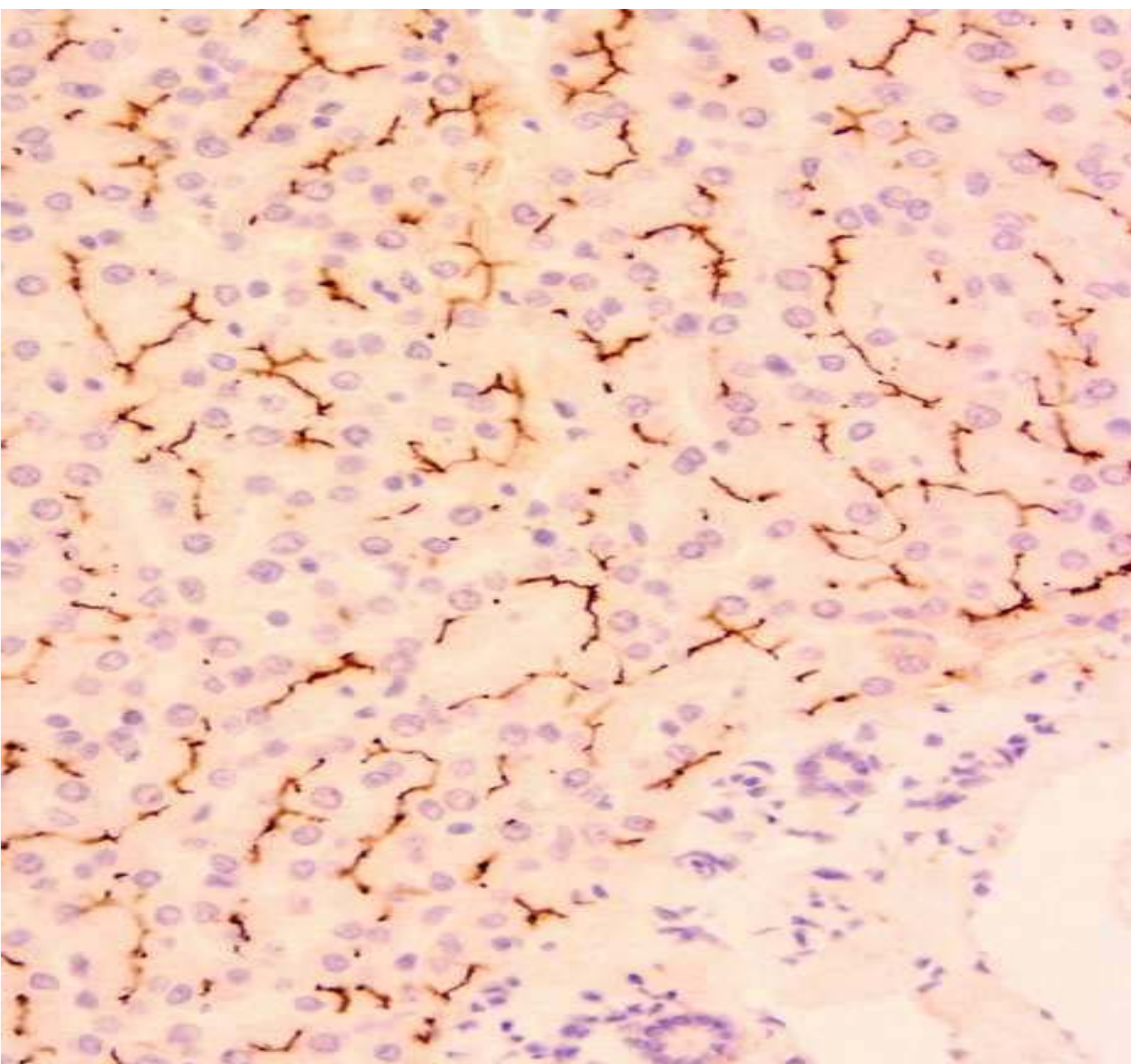
c.1763C>T	p.Ala588Val	15	No	Adj Walker B			Y		A ^A A A A ^A	2	70, 89
c.2272G>C	p.Gly758Arg	19	No	NBF / TM	Y	Y	Y		G G G G G G	1	27
c.2296G>A	p.Gly766Arg	19	Yes	TM7	Y	Y	Y		G G G G G G	1	28
c.2494C>T	p.Arg832Cys	21	Yes	IC3	Y	Y	Y	Y	R R R R R R	2	29, 90
c.2576C>G	p.Thr859Arg	21	No	IC3	Y	Y	Y	Y	T T T T T T	1	43
c.2842C>T	p.Arg948Cys	23	Yes	IC4	Y	Y	Y	Y	R R R R R R	2	42, 71
c.2935A>G	p.Asn979Asp	23	No	TM11		Y			N N N N H H	1	30
c.2944G>A	p.Gly982Arg	23	Yes	TM11	Y	Y	Y		G G G G G G	4	31, 37
c.3086C>A	p.Thr1029Lys	24	No	TM12	Y	Y	Y	Y	T T T T ^M V	1	91
c.3329C>A	p.Ala1110Glu	25	Yes	Adj Walker A	Y	Y	Y		A ^A A A A ^A	2	47, 72
c.3382C>T	p.Arg1128Cys	25	Yes	Adj Walker A	Y	Y	Y	Y	R R R R R R	1	32
c.3457C>T	p.Arg1153Cys	26	Yes	NBF2	Y	Y	Y	Y	R R R R R R	4	73
c.3458G>A	p.Arg1153His	26	Yes	NBF2		Y	Y		R R R R R R	4	33, 34,
c.3460T>C	p.Ser1154Pro	26	No	NBF2				Y	S ^S S S A ^A	1	50

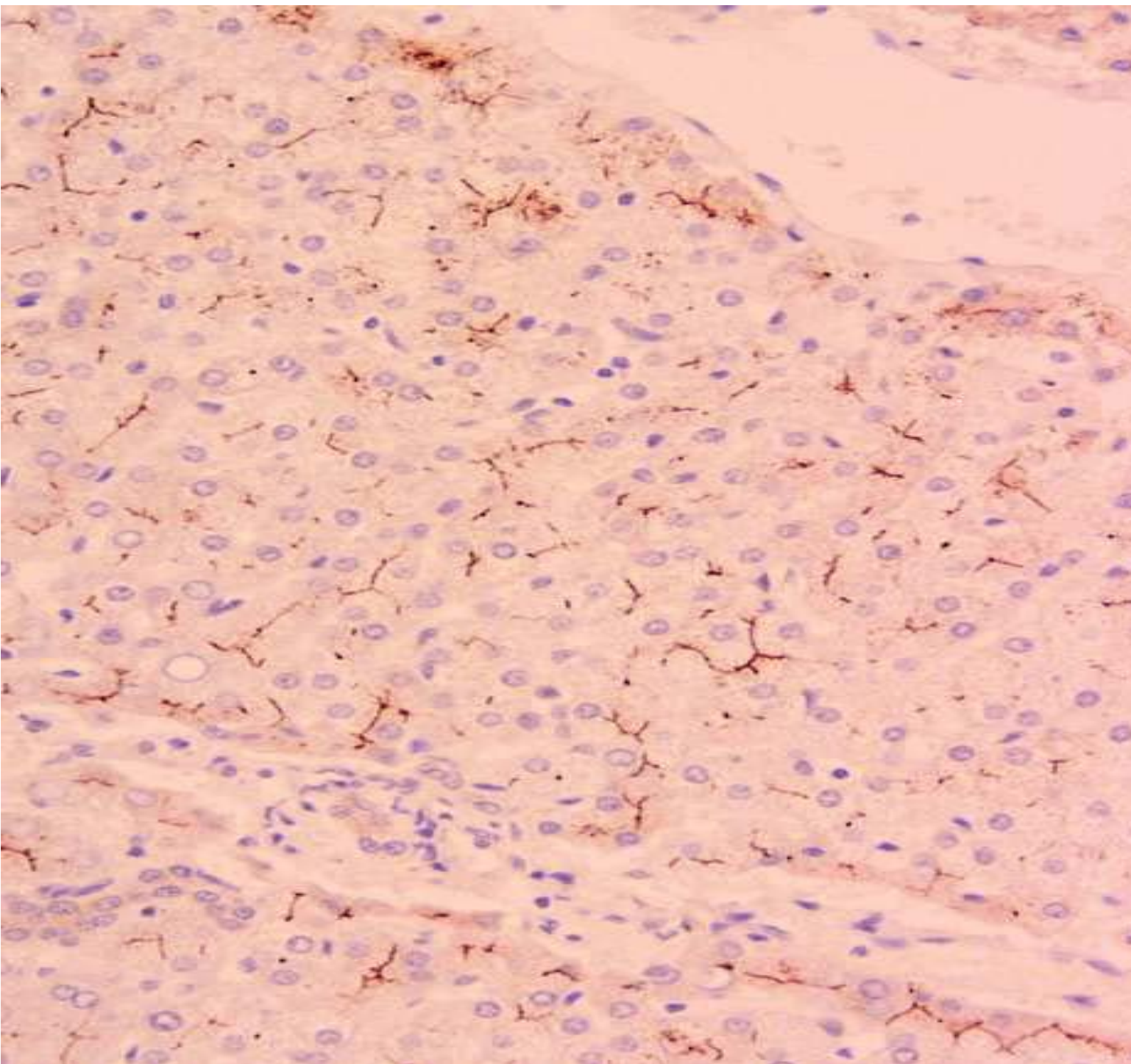
c.3628A>C	p.Thr1210Pro	27	No	Adj ABC				Y	T	T	T	T	T	T	1	35
c.3691C >T	p.Arg1231Trp	27	Yes	ABC / Walker B		Y	Y		R	R	R	R	R	R	1	92
c.3692G>A	p.Arg1231Gln	27	Yes	ABC / Walker B		Y			R	R	R	R	R	R	2	48
c.3724C>A	p.Leu1242Ile	27	No	Walker B					L	L	L	L	L	L	1	45
c.3892G>A	p.Gly1298Arg	28	No	NBF2	Y	Y	Y		G	G	G	G	G	G	1	49

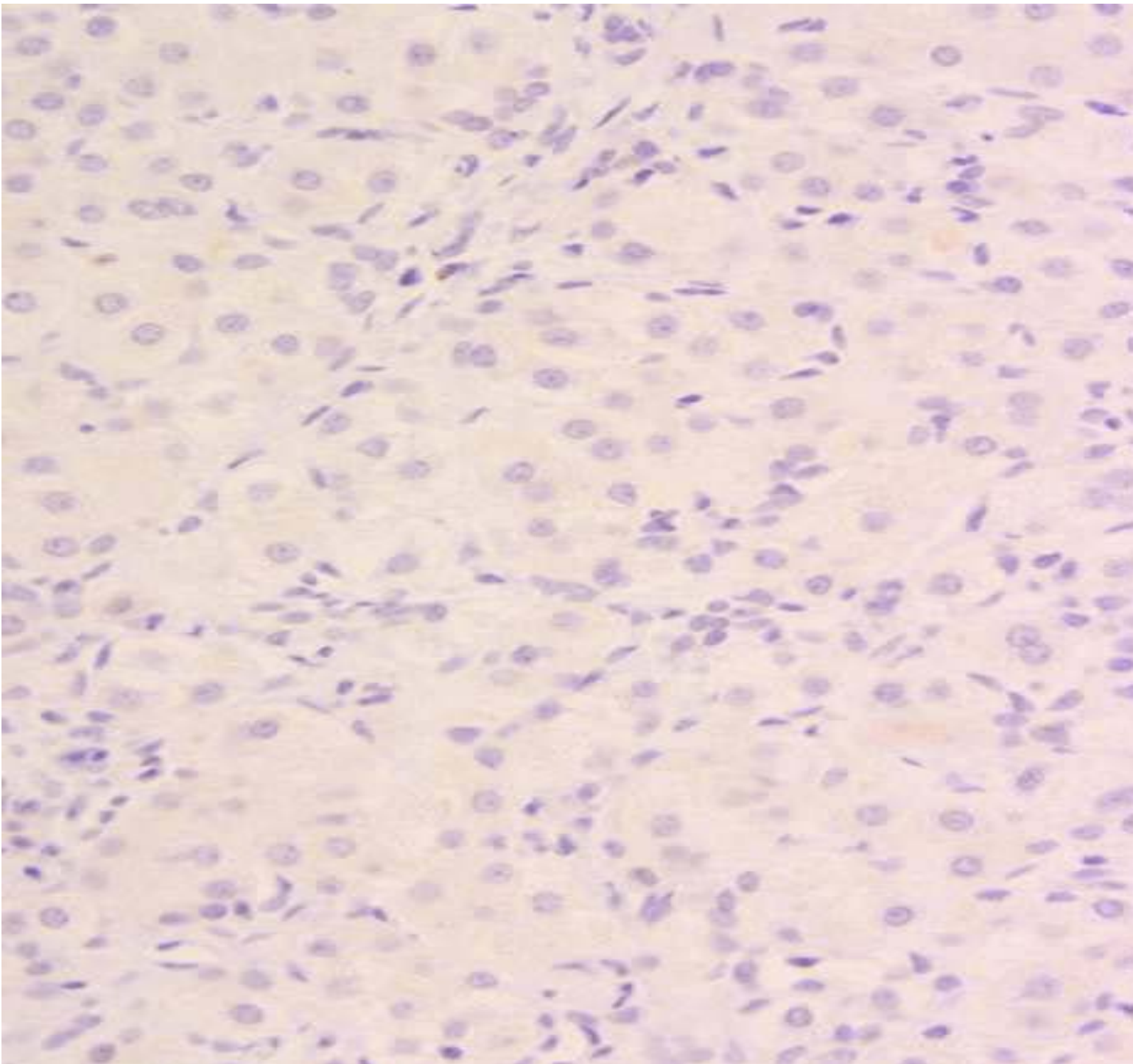
Table 3: Missense mutations in *ABCB11*

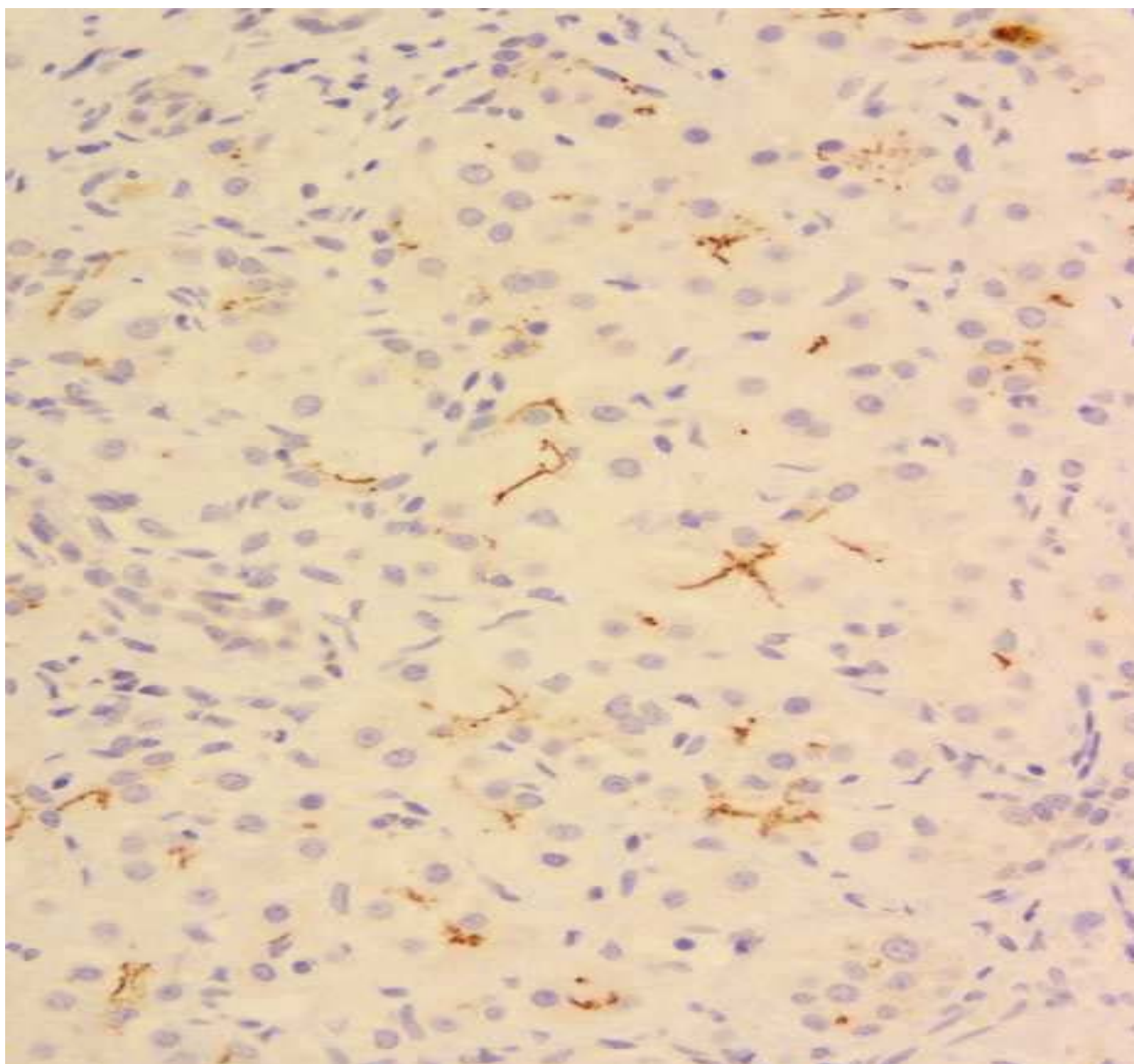
Biliary Canaliculus

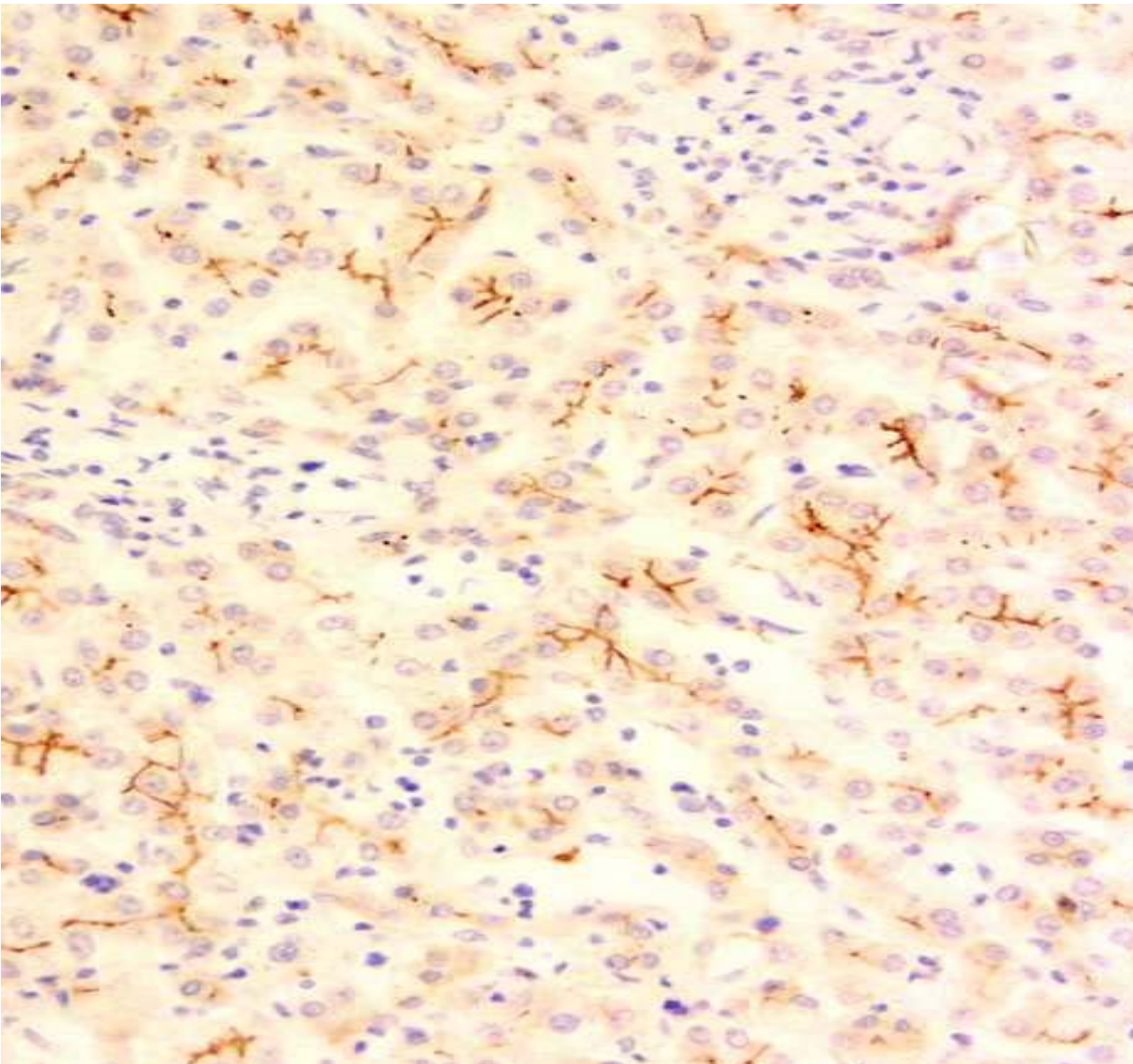


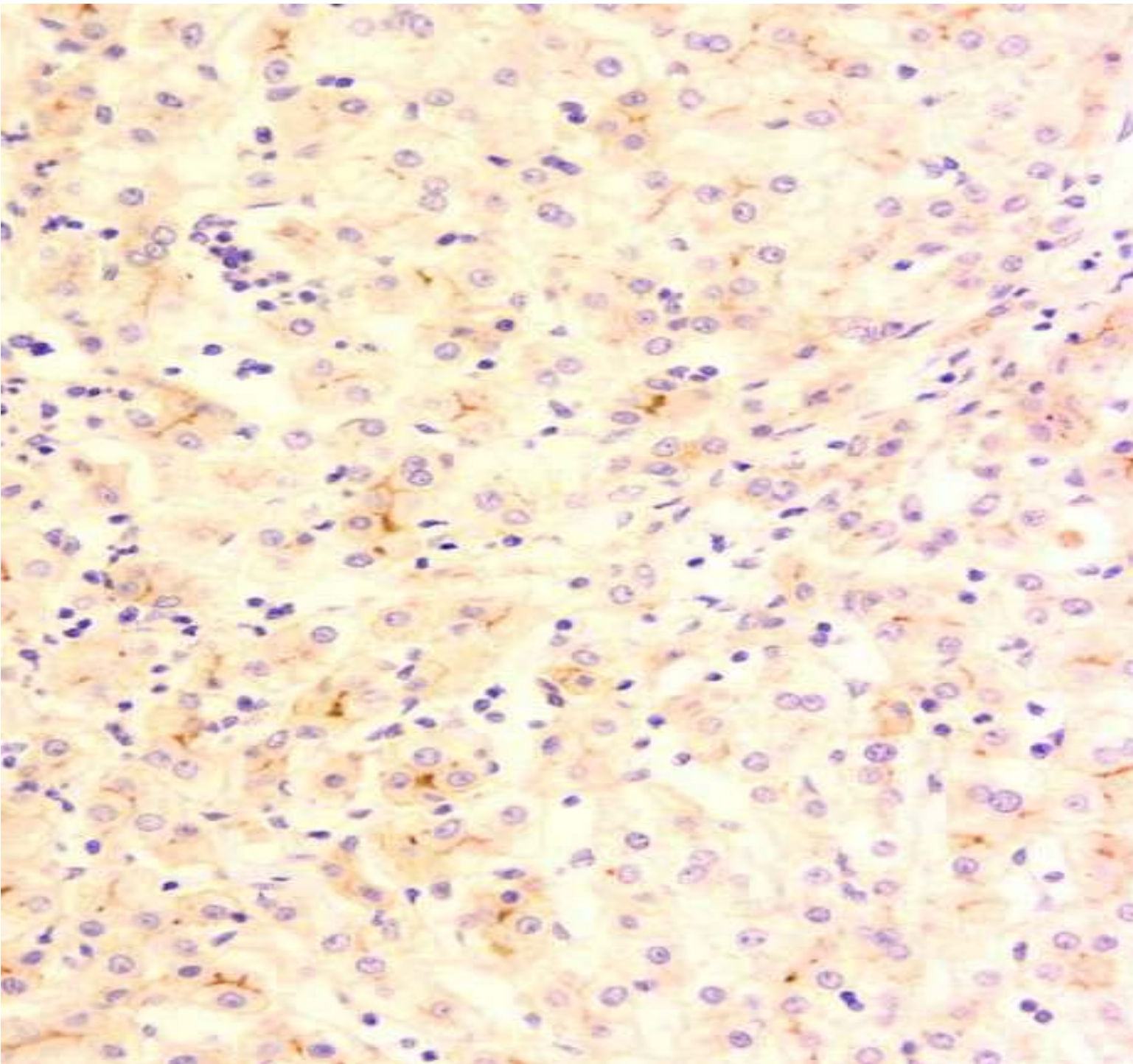


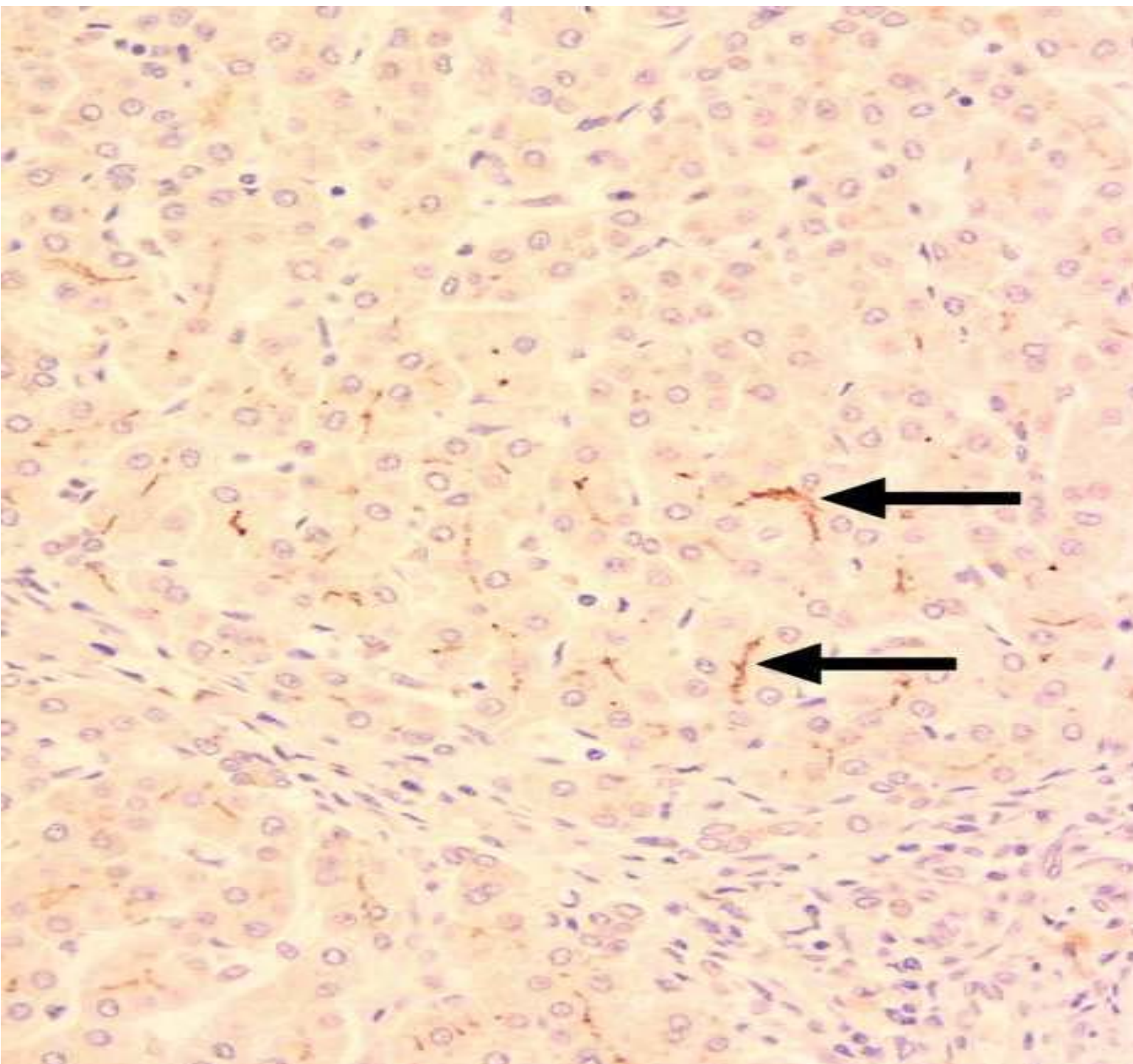


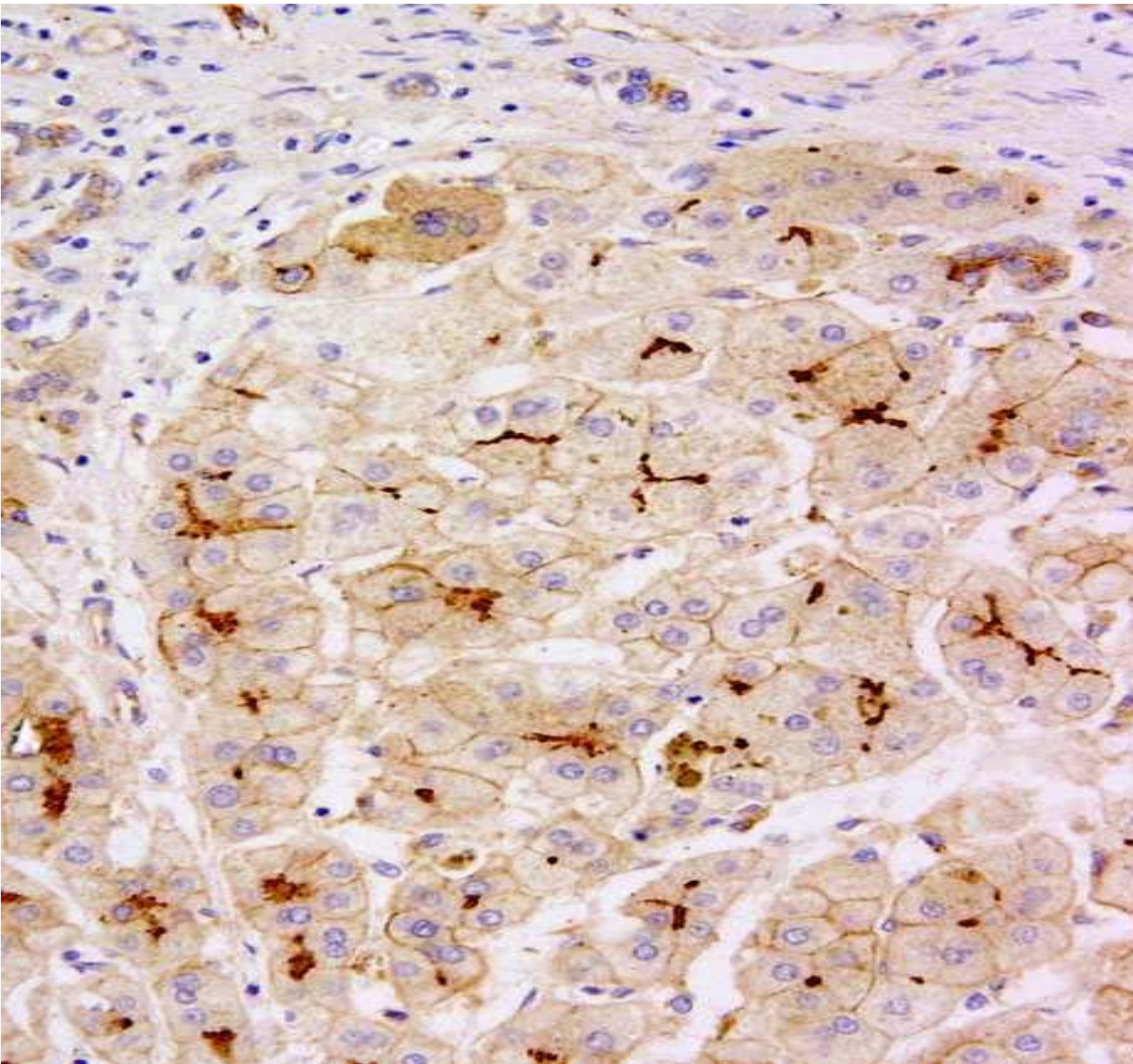












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